Thesis Summary of the PhD Dissertation

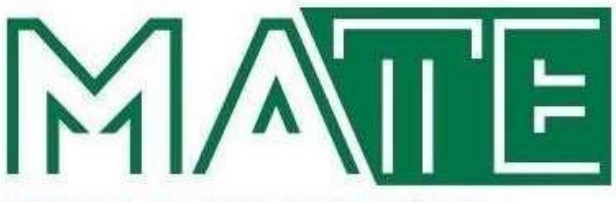
DYNAMIC CHANGES IN SPERM CHROMATIN STATUS IN SEASONALLY BREEDING MAMMALS

By: Ariuntungalag Javkhlan

Supervisors:

Prof. Dr. Nagy Szabolcs Tamás

Dr. Bodó Szilárd



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1. INTRODUCTION

Breeding soundness evaluations (BSEs) are an essential step in ensuring the reproductive success of all species of animals (stallion, ram, bull, and domestic etc.,). It is an examination that evaluates the physical and reproductive soundness of livestock for breeding purposes (Watts et al., 1997). There are several reasons to use BSE: i) it confirms that the animal is healthy and free from any physical abnormalities ii) to identify any reproductive issues in animals that can affect their ability to reproduce. This is especially important for farmers and breeders since it can prevent economic loss (Palmer, 2005, 2016). Additionally, it can help to prevent or stop sexually transmitted diseases (Barth et al. 2004). All of the above-mentioned leads to sustainable management for cryopreservation of animal genetic resources which is important to maintenance of biodiversity, ensuring food security, and improving population livelihoods globally.

Male fertility is a key factor of reproductive biology of domestic animals and therefore, has been considered an essential economic necessity in breeding strategies for successful animal production (Salisbury et al., 1978). Evaluating spermatozoa quality is an important tool for evaluating the propagative capacity of a species and to determine the efficacy of methods that could be developed to increase fertility in bulls, stallions and rams. Research of domestic animals indicated that the early embryonic death is often the result of DNA fragmentation seen as nuclear defects in the spermatozoa. Abnormalities of the sperm DNA can cause decreased fertility and poor embryo quality and decreased pregnancy risk.

Kruger *et al.* (1993) designed a computer-assisted sperm morphometric analysis (CASA) for more accurate evaluation of human sperm morphology. This method was later successfully adopted for the assessment of the physical characteristics of some animals. Gravance et al. (1996) successfully evaluated the head of the spermatozoa from bulls utilizing CASA. They successfully evaluated sperm head morphology of rabbits and sheep (Gravance et al., 1995:1998) and stallion (Ball and Mohammad et al., 1995).

The precision of the CASA system requires calibration to each species based on technique, smear preparation, and classification (Banaszewska *et al.*, 2015). The precision of the assessment of the physical characteristics of the sperm depends on the method of staining as this impacts the shape and dimensions of the head (Hirano *et al.*, 2001). In many laboratories, different staining

technics are used. Because of the chemical make-up of the reagents used in the staining process, variable effects on the stained cells can occur as well (Banaszewska *et al.*, 2015).

Utilization of the Papanicolaou staining technique sperm morphology assessment takes more time and requires the use of plethora of chemicals and processing stages (>20) (Banaszewska *et al.*, 2015). The eosin-negrosin staining method is faster and easier, however, it is not appropriate for cryopreserved sperm and is slightly hypotonic (Kumar *et al.*, 2019).

A necrotic swelling and larger sperm head appear in sperm that have succumbed prior to smear preparation and staining because of damaged plasma membranes and acrosome deterioration (Kumar et al., 2019). Additionally, this staining method is rapid, inexpensive, and the most practical tool.

Feulgen staining technique has been used in prior research on spermatozoa morphology (Wishart et al., 1988). Along with detecting abnormalities of the morphometry of the nucleus, this technique also identifies aberrations of the sperm chromatin (Barth and Oko, 1989). Our laboratory has successfully implemented this staining technique on several domestic and wild animal species in combination with different image analysis platforms.

2. OBJECTIVES

Assisted reproductive technologies (ART) provide useful tools to save the genetic diversity of endangered and other species. Using gamete cryopreservation as an ART tool to create gene banks has been used since the 1990-s. Sperm storage technologies developed for domestic animals can be applied to different species providing a useful conservation tool.

It has been long known that male fertility is related to sperm morphology (Williams and Savage, 1927). Spermatozoa morphology is a routine part of the BSE of domesticated animals used for breeding (Barth and Oko, 1989). Differences in sperm size may reveal hidden reproductive strategies such as postcopulatory sperm competition or cryptic female choice (Kahrl et al., 2021). The Feulgen staining technique was used to label chromatin in order to minimize the effect of staining and membrane status. This procedure has been modified and successfully performed on different species, both domestic and wild. We have successfully adapted and used

this staining protocol on different domestic and wild animal taxa in combination with different image analysis platforms.

Accordingly, the main goals were to:

i. evaluate the nuclear condensation status of sperm collected *post-mortem* from Przewalski's horse via Feulgen staining and light microscopy.
ii. cryopreserve spermatozoa from different species such as sheep (Hungarian native breed sheep), cattle (Simmental bulls) and domestic yak (wild and domestic species with special importance in Mongolia).
iii. to measure the sperm head, midpiece and flagellum length of *post-mortem* collected and cryopreserved Przewalski's horse spermatozoa using the free Sperm Sizer software.

According to the IUCN Red List, the Przewalski's horse (*Equus ferus*) is considered to be endangered. Due to the gap in available information, our goal was to measure the sperm head, midpiece and flagellum length of post mortem collected and cryopreserved Przewalski's horse spermatozoa using Feulgen staining and the free Sperm Sizer software (McDiarmid et al., 2021). Through repeated measurements, we established the repeatability of this staining protocol and morphometric analysis. Major parts of the thesis were the use of bull and ram spermatozoa to perform the Feulgen staining technique on a seasonal, local sheep breed, to study the reliability of the technique on Simmental bulls, and to show its diagnostic value in this pilot research. Another important topic relevant to Mongolia was a preliminary study on the threatened domestic yak species (*Bos grunniens*). We aimed to evaluate the nuclear condensation status of cryopreserved domestic species spermatozoa via Feulgen staining technique and light microscopy.

3. MATERIALS AND METHOD

3.1 Ram semen

3.1.1 Study location

The present study was performed at the National Agricultural Research and Innovation Center, Research Institute for Animal Breeding, Nutrition and Meat Science, Herceghalom, Hungary. Portions of the work were affiliated with the University of Pannonia. In this study, 78 breeding rams were chosen from Hungarian Sheep and Goat Breeder's Association for the Gene Conservation Program. Samples were transported from different farms in Hungary to the Experimental Farm and Artificial Insemination Station of the Research Institute between September 2014 and April 2017. The rams represented four breeds and they were housed together in a shelter. They had *ad libitum* access to water, grass and hay. Some of the rams were collected at the end of or after the breeding season. Approximately, 30 percent of the rams were able to be trained to an AV. A total of 24 rams (6 Hortobágyi Racka, 7 Cikta, 8 Tsigai, 3 Milking Tsigai) were able to be successfully collected in the gene conservation program. In this study, 17 rams were used during the breeding season and 20 rams were used out of season.

3.1.2 Semen collection

In the first 30 days of quarantine, each ram was examined by a veterinarian. Jugular blood samples were collected from all of the rams for infectious disease surveillance tests including B. ovis, B. melitensis, Border disease, and Bluetongue. After quarantine, each ram was trained to use an AV. At the onset of the semen collection period, a sperm sample from each ram was bacteriologically examined by at the National Food Chain Safety Office in Hungary. Males whose semen was pathogen free were utilized for semen collection. An AV (IMV technologies, L'Aigle, France) was utilized to collect semen sample in the presence of an ewe in estrus one or two times a week until 200 doses of sperm were collected, frozen and stored.

One or two emitted semen samples were collected every day with a 15 – 30 min interval between each collection. Each sample was held at 37ºC until completion of the semen analysis. A spectrophotometer (Accucell, IMV technologies, L'Aigle, France) was used to measure the spermatozoa concentration after the semen volume of the ejaculate was determined. Subsequently, the total number of spermatozoa per collection was estimated. Phase contrast microscopy (Olympus BX-51, Olympus Life and Material Science Europa GmbH, Hamburg, Germany) was

used at 50× magnification to assess sperm motility in undiluted semen and scored on a scale of 0-5. A sample was diluted with semen extender at 1:50 and at 1:100 dilution rate and viewed with a phase contrast microscope at 200×magnification. Each ejaculate contained greater than 2×10^9 cells per mL and had over 75% motility. Freezing was conducted manually in a Styrofoam box with 4 cm of liquid nitrogen for 8 min. After that the frozen straws were plunged into liquid nitrogen, and each semen straw was placed in a well-labeled goblet for permanent storage in the Gene Bank. In the assessment of frozen and thawed semen samples, motility analysis of each semen sample kept in the Gene Bank was accomplished and three representative frozen samples (one each from the beginning, the middle, and the end of the semen collection period) of each ram were chosen for further measurement.

3.2 Przewalski's stallion semen

3.2.1 Sampling location

The stallions were foaled in Munich as a descendent of highly inbred A-line, and all of them arrived in Hortobágy, Hungary in 2012. In the core area of the Hortobágy National Park, the Pentezug Wild Horse Reserve houses Przewalski's horses and "reconstructed ancient stallions" in semi-wild conditions. This area is part of Zone A, which means that it is an area closed to visitors and no agricultural activities are allowed. From here, selected individuals are transferred from Pentezug to the Wildlife Park, where guests can also admire them. Pentezug has been an officially designated reserve since 1997. The Pentezug reserve was expanded to 3,000 hectares in 2015, surrounded by a special electric fence that keeps Przewalski's horses and wild boars at bay due to their size, but wild animals such as foxes, rabbits and roe deer can roam freely.

3.2.2 Semen collection

Post mortem five Przewalski's stallions were used for this study. In 2014, 11 Przewalski's horses were culled from the Pentesug herd for various reasons (high inbreeding factor, prolonged illness, abnormal behavior). After dissection of the testis and epididymis, measurement of weight and dimensions, epididymal spermatozoa were obtained from the five stallions by air blowing, retrograde sperm diluent rinsing or cutting. Also, all parameters of testicle and epididymal from five culled stallion were recorded. Subsequently, cryopreserved semen was

transferred to the laboratory. Automatic filling apparatus was used to fill into 0.25 ml straws. Finally, all samples were transferred into a liquid nitrogen container for storage and further measurements.

3.3 Simmental Bull semen

3.3.1 Sampling area

Sperm samples from twenty Hungarian Simmental bulls were provided by the Hungarian Simmental Breeder for this project. The Feulgen staining technique was studied to explore its value as a diagnostic technique.

3.3.2 Semen collection

A total of 20 cryopreserved single-dose encoded straw samples were received in our laboratory on the Georgikon Campus (Keszthely). The Association of Hungarian Simmental Breeders (AHSBs) provided frozen semen according to the Animal Husbandry directive Decree No. 45/2019. (IX.25.). Semen extender was used to dilute each sample. Diluted semen in AHSBs were packed in straws. The information for all samples was recorded, including name, packer numbers and date, etc. The Association of Hungarian Simmental Breeders categorized 20 spermatozoa samples as "Good" and "Bad" based on their reproductive performance criteria. Twelve sperm samples were classified as bad, and 8 as good. All samples were kept in liquid nitrogen until evaluated.

3.4 Mongolian domestic yak

3.4.1 Sampling area

The semen samples were provided by Semen laboratory of the National Gene Bank Center of Livestock, Mongolia. Semen samples were collected between Sept and Oct, 2019. Three younger and two older yak bulls were chosen based on physical characteristics such as body condition, weight, and shape and so. All domestic yak bulls were coming from Ikhtamir county, Arkhangai province. Each bull was fed good quality green hay, starting on the 25th of the August.

3.4.2 Semen collection

The semen of the domestic yaks was collected from 26th of September and 2nd of October. The artificial vagina (AV) was used. Equipment condition was equilibrated for heat of the vagina, pressure, and slipperiness. The use of the AV is very complicated to collect semen from each domestic yak. Because it was not natural way. The semen was collected successfully from two domestic yak. Whereas, remained three were not able to successfully collect semen samples. The Triladyl extender was used. Spectrophotometry was used to determine the semen volume of all domestic yak, and the number of doses and code of each straws was noted. Each semen samples were kept at 37ºC prior to measurement. Each semen from domestic bull were stained in Mongolia and then prepared slides were transport to the laboratory on the Georgikon Campus (Keszthely) from Semen Laboratory of the National Gene Bank Center of Livestock, February, 2023.

3.4 *Feulgen staining*

Straws were thawed in a 37°C water bath for 30 sec, and then smears were made on Superfrost slides. A few drops of each sperm suspension were dropped on clean glass slides and spread slowly from one end of the slide to the other using another clean slide. Air-dried smears were stained with Feulgen staining kit (Merck, cat.no. 1079070001) according to the protocol suggested by the Manufacturer, with modifications. Slides were placed in 5 mol/L HCl solution (50 min) at 22ºC in a Hellendal jar, then rinsed for 5 min under running tap water. Slides were then transferred into Schiff's reagent and kept in the dark for 1 hr. Slides were rinsed with sodium bisulfite solution for 3 min, then under running tap water for 10 min. Slides were allowed to dry at room temperature then covered with Merck Entellan and coverslip.

3.5 Morphology assessment

The sperm morphology assessment is widely used, and a part of breeding soundness evaluation (BSE). Considering the classification of the sperm abnormalities, sperm head, body, tail are most important criteria. For instance, considering the classification of spermatozoa abnormalities, the normal spermatozoa tail is uniform in observed, whereas spermatozoa head shapes are different, it is from short and broad to narrower and elongated. Therefore, microscopic

evaluation is most important to identify the morphological abnormal spermatozoa (proximal, cytoplastmic droplet, loose head, and abnormal acrosomes, nuclear pouches, abnormal midpiece, simple ciled tails, and so). Phase-contact microscope at 1000 × magnification with immersion objective was used to assess 500 spermatozoa in stained slides.

3.6 Morphometric assessment

The fully equipped Olympus equipment (the phase-contrast Olympus microscope (BX43) equipped with an Olympus DP26 digital camera Olympus Stream image acquisition software) was used to take digital photo at 1000 × magnification. Spermsizer free software (version 1.6.6.) was used to examine digital photos. It was measured twice. To examine the spermatozoa, users selected key points with left bottom of the computer mouse as follows: i) to start from top of the head to beginning of the midpiece, ii) to start from beginning of the midpiece to beginning of the tail, and iii) to start from beginning of the tail to end of the tail (McDiarmid et al., 2021). The measurement length in the digital images are expressed in pixel, and the measurement are saved as a CSV file. It can be transferred from pixel to micrometers.

3.7 Chromatin condensation

Chromatin condensation is a part of the BSE and it is a unique method. After staining, it was covered with Merck Entellan and coverlip. 100 spermatozoa per slides were assessed on digital images and classified as normal and abnormal chromatin condensation.

3.8 Data analysis

In Native Hungarian rams, a Shapiro-Wilk test was used to check normality. Two-way Analysis of Variance (ANOVA) was used to analyze the effects of breed (Tsigai, Cikta, Racka). The Tukey post hoc test was used to separate means.

In the Przewalski's horse, we analyzed the repeatability of sperm size measurements in pixels (head, midpiece, tail, total length) with rptR package (Nakagawa and Schielzeth, 2017). We calculated the size to μm from pixels (1μm was 15 pixels). The mean sperm lengths were calculated, and then the effect of the background variables on sperm sizes were tested. We used

linear models, where one of the sperm sizes was the response variable and one of the background variables (left or right testis size, age, testosterone in 2015 or in 2016) was the fixed effect. There were 20 models in this structure, and we used only 1 response variable or 1 fixed effect due to the low sample size. We corrected the p-values with Bonferroni-Holm methods as a control for multiple comparisons.

We calculated the ratio of the abnormalities in head, tail and chromatin by calculating the number of abnormal cells divided by the total number of cells within the particular ejaculate. We tested the effect of background variables on abnormalities using linear models. In these models, one type of abnormality (head, tail or chromatin) was the response variable and one of the background variables was the fixed effect. There were 15 models in this structure, and we used only 1 response variable or 1 fixed effect due to the low sample size. We corrected the p-values with Bonferroni-Holm methods as a control for multiple comparisons. In the domestic yak, a Shapiro-Wilk test was used to check normality and description. The Tukey post hoc test was used to separate means.

4. RESULTS

4.1 Ram semen

Considering the morphological parameters, the normal mean percentage of Racka was higher than that of the Citka and Tsigai breeds. In the abnormal morphology, percentage of the tail bent was higher in each breed. The higher tail-bent was observed in Tsigai, as followed by Citka and Racka breed. High amount of the abnormal distal midpiece reflex was appeared in Tsigai, but lowest was found in Racka.

Considering the spermatozoa morphometric assessment, the standard deviation of the Citka breed was significantly higher than that of Tsigai and Racka breeds. Each parameters of present study were not affected by season. Whereas, average and spermatozoa length observed significant season and breed interaction. Abnormal chromatin condensation was detected in Tsigai and Citka breeds. In the Citka breed, higher percentage of chromatin status was observed.

4.2 Przewalski's stallion semen

Considering the individual head, midpiece, and tail, each stallion spermatozoa length was significantly observed. In the mean head and midpiece length, ID8 had longest, but ID1 has wider boxplot of head length. Lowest mean head length was found in ID7. The lower midpiece length was appeared in ID1, whereas, the highest parameter of mean tail length in ID1 and ID3. A significant negative correlation was observed between age and head abnormality. A significant correlation between chromatin abnormality and the background variables was not found. In the abnormal head percentage of the Przewalski's horse, it was ranged from 3% to 8%. Highest percentage was observed in ID2, whereas lowest percentage was found in ID7. In the tail, it was ranged between 24.7% and 46.6%. Highest was found in ID1, whereas, lowest percentage appeared in ID7. Considering the percentage of the chromatin status, it was ranged from 2% to 8%. The average standard deviation of the abnormal chromatin was 2.4±1.7%. Highest percentage was found in ID7, but lowest was observed in ID2.

4.3 Simmental bull semen

The mean head length of the “good” and “bad” Simmental bulls ranged from 7.2 µm and 7.1 µm. The standard deviation of the “good” Simmental bulls was significantly lower than that “bad” Simmental bulls. Considering the morphology assessment, the average percentage of the abnormal tail-bent was 2.5% in “good” all bulls. In the “bad” bulls, percentage was 4.7%. The average percentage of the no head was higher in “bad” bulls than “good” bulls. The average percentage of the dag defect was two times higher in “bad” bulls than those “good” bulls. In the coiled tail, it was not detected in “bad” bulls. Surprisingly, it was observed in “good” bulls. In the average percentage of the pyriform, it was three times higher in “bad” bulls. In the bad bulls, average percentage of the distal midpiece reflex was 9.6%, but in the “good” bulls, it was 3.6%, respectively. Considering the abnormal chromatin condensation, it was not found in two categorized bulls.

4.4 Domestic yak semen

Considering the morphometric parameters, the maximum value of the head length was 8.2µm. But the minimum of the head length was 7.1 µm. The mean body length of the spermatozoa was 12.6 µm. In the tail length, mean value was 48 µm, the max was 56.7 µm. whereas, minimum tail length was 33.1 µm. The number of bull spermatozoa ranged from 33 and 70. The normal percentage was 71%. Higher percentage of the abnormal tail-bent was found (15.2%). In the detached head, coiled tail, and no head, it was 5.6%, 4%, and 2.8%, respectively. Abnormal spermatozoa nuclei were not observed in case of our study.

5. TOTAL SUMMARY

Male fertility is a crucial factor of reproductive biology of domestic animals. It has been considered an essential economic trait in breeding strategies for the successful establishment (Salisbury et al., 1978). Most studies reported that spermatozoa quality is an important assessment of the reproductive capacity of animal species and to determine the efficacy of methods that could be developed to determine fertility in bulls, stallions, rams and other species. Sperm quality is

directly connected to sperm morphology and morphometry. Morphology assessment is evaluated on concentration, volume, and viability. Due to the scarcity of spermatozoa in some species, it is difficult to accurately interpret data or achieve statistical significance.

Ram spermatozoa assessment

- Feulgen stained smears were photographed under a phase contrast microscope (Olympus BX-51), using 40x objective with cellSense Standard Imaging Software by Olympus. There were not any abnormalities in photograph.
- In the spermoatoza morphology on the stained smear, there were not any abnormalities in photograph. The effect of Cikta and Tsigai breeds on spermatozoa head morphometry showed that rams differed significantly ($p<0.005$) on nucleus length parameters.

Przewalski's horse spermatozoa assessment

- The semen quality parameters of five stallions passed routine post-thaw quality criteria. The semen doses were 100 million total sperm per ml, and minimum 35 percent progressive motility.
- The percentage of the abnormal head of the Przewalski horse spermatozoa ranged from 3% to 8% in case of this study. The percentage of abnormal tails of Przewalski horse spermatozoa ranged between 24.7% and 46.6%. The highest percentage found was in ID1 (46.6%), whereas the lowest percentage was in ID 7 (24.7%), respectively. ID 8 was 42.7 %, followed by ID 2 (36.1%), and ID 3 (30%).
- Individual differences exist in the morphometry measurement of the Przewalski horse spermatozoa. In the mean head and midpiece length, ID 8 had longest, whereas ID 1 has wider boxplot of head length. Lowest mean head length was observed in ID 7. In ID 2 and ID 3, mean head length was quite similar. The lowest midpiece length was found in ID 1. The highest parameter of mean tail length in ID 1 and ID 3 were followed by ID 2, ID 7, and ID 8. The total mean length was 53.83 µm (sd = 2.00) for the species. None of the background variables had an effect on the sperm sizes (all p's > 0.23).
- The percentage of the chromatin status in Przewalski horse ranged between 2% and 8%, the average (±SD) % of abnormal chromatin was 2.4 ±1.7%; (2, 5, 3, 1, 1% in individual

stallions). The highest percentage was found in ID 7, the lowest was determined in ID 2. In the ID 1, ID 2, and ID 3 percentages were similar. In the Przewalski's horse we demonstrated individual differences in sperm domain sizes.

Simmental bull spermatozoa assessment

- The percentage of the normal measurement of the "good" and "bad" Simmental bull spermatozoa was evaluated. The percentage of the normal measurement in "good" bulls ranged from 47.2% to 92%. The mean percentage of the normal sperm in "bad" and "good" bulls ranged between 62.3% and 80.4%, respectively.
- The percentage of the abnormal head, midpiece, and tail of measurement of the good and bad Simmental bull spermatozoa was evaluated. The average percentage of the abnormal tail bent in total good Simmental bulls was 2.5%, whereas percentage of the abnormal tail bent in total bad bulls was 4.7%, respectively. The average percentage of the no head in bad bulls was higher than in those good bulls. The average percentage of the pyriform in bad bull was more than three times that in those good bulls. In the tail bent in good bulls, a higher percentage appeared in ID 20 (7.2), whereas in bad ID 11, it was 17.5%. The high percentage of the nuclear crest, coiled tail, pyriform, and distal midpiece reflex in good ID 2, 15, 1, and 13 were 5%, 4%, 1.4%, and 9%, respectively. Whereas, high percentage of the nuclear crest, pyriform, and distal midpiece reflex in bad ID 18, and 8 were 6.1%, 3.4, and 75%, respectively.
- Simmental breed bulls spermatozoa head with Feulgen staining under phase contrast microscopic measurement was evaluated. In this study, we only measured head length in good and bad Simmental bulls. The mean head length of good and bad Simmental bulls ranged between 7.2 and 7.1. The SD of good Simmental bulls (0.32) was significantly lower than that of bad Simmental bull (0.37) ($p< 0.05$)

Mongolian domestic yak spermatozoa assessment

- The percentage of abnormalities in different parts of the Feulgen-stained Mongolian yak spermatozoa was evaluated. Five straws from Bull-1 were used in a case study as a pilot project. The number of normal sperm from a yak bull ranged between 33 and 70. The normal percentage of bull spermatozoa was 71%. The higher percentage of the abnormal

tail bent (15.2%) of the Mongolian yak spermatozoa was found in this study as the following: detached head (5.6%), coiled tail (4%), no head (2.8%), and Dag defect (1%). The lowest percentage observed was the nuclear crest. However, pyriform and distal midpiece reflexes were not detected at this time.

- Mongolian yak bull head, body, and tail spermatozoa with Feulgen staining under phase contrast microscopic measurement was evaluated. The mean head length was 7.7. The maximum value of the head length was 8.2. Whereas the minimum of the head length was 7.1. The mean length of the spermatozoa body was 12.6. The maximum and minimum values of the spermatozoa body were 15.6 and 8.6, respectively. In the length of the spermatozoa tail, mean value was 48. The max was 56.7. Whereas, in the tail length, it was 33.1.

6. NEW SCIENTIFIC POINTS

I. The current study observed that the Tsigai and Cikta breeds have differences in sperm head morphology. However, no differences were found in Racka breed sperm morphometric traits.

II. I found differences in intra-male sperm head variance in Simmental bulls, and these differences can impact sperm quality.

III. In Przewalski's horse, I demonstrated individual differences in sperm domain sizes.

IV. This was the first time Mongolian domestic yak bull sperm was examined for morphometry, morphology, and chromatin status with the Feulgen staining under light microscopy.

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8. SCIENTIFIC PUBLICATIONS OF THE AUTHOR

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